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ORIGINAL ARTICLE

Novel Cathelicidins with Potent Antimicrobial, Biofilm Inhibitory, and Anti-inflammatory Activities from the Frog *Fejervarya multistriata*

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Abstract Antimicrobial peptides (AMPs), a class of gene-encoded peptides, are the first line of immune system to defense microbial invasions in multicellular organisms. Cathelicidins are an important family of AMPs that have been identified exclusively in vertebrates. However, up to now, cathelicidins from amphibians are poorly understood. In the present study, we reported the identification and characterization of two novel cathelicidins (FM-CATH1 and FM-CATH2) from the frog *Fejervarya multistriata*. The cDNA sequences encoding FM-CATHs were successfully cloned from the constructed lung cDNA library of *F. multistriata*. Both of the cDNA sequences encoding FM-CATHs are 447 bp in length, and the deduced mature peptides of FM-CATHs are composed of 34 residues. Structural analysis indicated that FM-CATH1 and FM-CATH2 mainly assume amphipathic alpha-helical conformations. Antimicrobial and bacterial killing kinetic analysis indicated that both FM-CATH1 and FM-CATH2 possess potent, broad-spectrum and rapid antimicrobial potency. And cytoplasmic membrane permeabilization analysis indicated that FM-CATH1 and FM-CATH2 kill bacteria by inducing the permeabilization of bacterial membrane. Besides direct antimicrobial activities, FM-CATHs also exhibited significant inhibitory effect on the formation of bacterial biofilms at low concentrations below 1×MIC. Furthermore, FM-CATH1 and FM-CATH2 exhibited potent anti-inflammatory activities by inhibiting LPS-induced transcription and production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in mouse peritoneal macrophages. Meanwhile, FM-CATHs showed relatively low cytotoxic activity against mammalian normal and tumor cell lines, and low hemolytic activity against human erythrocytes. In summary, the identification of FM-CATHs provides novel clues for our understanding of the roles of cathelicidins in amphibian immune systems. The potent antimicrobial, biofilm inhibitory, anti-inflammatory activities, and low cytotoxicity of FM-CATHs imply their great potential in novel antibiotics development.

Keywords cathelicidin, *Fejervarya multistriata*, FM-CATHs, antimicrobial peptide, bacterial biofilm, anti-inflammatory

1. Introduction

Living in an environment surrounded by all sorts of pathogens, multicellular organisms are permanently under threats of diverse microbial infections (Zasloff, 2002). In order to successfully survive, they evolved powerful and complex immune system to cope with microbial invasions (Akira *et al.*, 2006). Animal immune system is composed of two branches, innate and adaptive immunity. Innate immunity is the first line of host defense against microbial infection, while adaptive immunity exerts the defense function through many different immune cells and antibodies (Tosi, 2005; Akira *et al.*, 2006). Antimicrobial peptides (AMPs) are a class of gene-encoded peptides that have been discovered from nearly all sorts of organisms (Zasloff, 2002). They are one key component of animal innate immune system and play critical roles in host immune response to microbial infections (Nakatsuji and

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Gallo, 2012; Zasloff, 2002; Radek and Gallo, 2007). They usually possess direct antimicrobial activities against bacteria, fungi, viruses, and even parasites (Nguyen *et al.*, 2011). Besides, some of them possess other functions, such as modulating immune responses (Hancock *et al.*, 2012; Scott *et al.*, 2007).

Cathelicidins are a family of important AMPs with multifunctions, which are found exclusively in vertebrates. The first cathelicidin named Bac5 has been found from bovine neutrophils (Gennaro *et al.*, 1989; Zanetti *et al.*, 1990). Then a large number of cathelicidins were identified from other vertebrates. Generally, cathelicidins are synthesized as precursors, with a structure including a N-terminal signal peptide (30 residues), a highly conserved cathelin domain (99–114 residues) and a heterogenic C-terminal mature peptide (12–100 residues) (Zanetti, 2004). After translation *in vivo*, the cathelicidin precursors were cleaved by proteases (such as elastases) to release the mature peptides, which exert the antimicrobial and immunomodulatory functions (Zanetti *et al.*, 2000; Zanetti, 2004). Most cathelicidins possess strong antimicrobial activities against a variety of microorganisms, including bacteria, fungi, virus, and parasites (Zanetti *et al.*, 2000; Zanetti, 2004). As a result, they exhibit a great potential in the development of novel peptide anti-infective agents. Besides direct antimicrobial activity, many cathelicidins also are actively involved in various phases of host immune modulation and disease resistance, such as chemoattracting and activating immune cells, inhibiting NADPH oxidase, promoting angiogenesis and wound healing (Brown *et al.*, 2011; Wei *et al.*, 2012; Scott *et al.*, 2002; Mookherjee *et al.*, 2006; Xiao *et al.*, 2006).

Until now, only six amphibian cathelicidins have been identified. They are cathelicidin-AL from *Amolops loloensis* (Hao *et al.*, 2012), cathelicidin-PY from *Paa yunnanensis* (Wei *et al.*, 2012), Lf-CATH1 and Lf-CATH2 from *Limnonectes fragilis* (Yu *et al.*, 2013), cathelicidin-RC1 and cathelicidin-RC2 from *Rana catesbeiana* (Ling *et al.*, 2014). In the present study, we report the identification and structure, function study of another two novel amphibian cathelicidins, named FM-CATH1 and FM-CATH2 from the frog *Fejervarya multistriata*.

2. Materials and Methods

2.1 Frog collection and tissue preparation Adult specimens of *F. multistriata* (n = 5, average weight = 40 g) were captured from Shouguang, Weifang, Shandong province, China (37.19°N, 118.32°E). No specific permissions were required for the sampling location/activity, and the current study did not involve endangered or protected species. After collection, the frogs were killed, and the lung was quickly removed and stored in liquid nitrogen until use.

2.2 cDNA library construction and screening of cDNAs encoding cathelicidins The tissue samples of *F. multistriata* stored in liquid nitrogen were grinded into powder and the total RNA was extracted using Trizol reagent (Life Technologies, CA, USA). The cDNA library was constructed by an In-Fusion SMARTer™ Directional cDNA Library Construction Kit (Clotech, Palo Alto, CA, USA). The experiment was carried out according to the kit instructions. The synthesized second-strand cDNAs was used as template for the following PCR-based cDNA cloning.

According to the highly conserved cathelin domain sequence of previously characterized cathelicidins, an antisense primer (5'-WSCRCAGRYCTTCACCTCC-3') was designed and coupled with a sense primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') designed according to the SMARTer V Oligonucleotide primer in the kit to screen the 5' fragments of cathelicidin cDNAs. The PCR procedure was: 5 min of denaturation at 94˚C; 30 cycles: denaturation at 94˚C for 30 s, primer annealing at 57˚C for 30 s, extension at 72˚C for 1 min. The last cycle was followed by an extension step at 72˚C for 10 min. The PCR product was purified by gel electrophoresis, cloned into pMD19-T vector (Takara, Japan) for sequencing.

According to the obtained 5' fragments of cathelicidin cDNAs, a sense primer (5'-ATGAAGGTCTGGCAGTGT GTGCTC-3') was designed and coupled with an antisense primer (5'-TACGCGACGCGATACGCGAAT-3') designed according to the 3' IF SMARTer CDS Primer in the kit to screen the full length cDNAs encoding FM-CATHs. The PCR procedure was: 5 min of denaturation at 94˚C; 30 cycles: denaturation at 94˚C for 30 s, primer annealing at 56˚C for 30 s, extension at 72˚C for 1 min. The last cycle was followed by an extension step at 72˚C for 10 min. And the PCR product was sequenced as described above.

2.3 Multi-sequence alignment and phylogenetic analysis Cathelicidin sequences used for multi-sequence alignment and phylogenetic analysis were obtained from the protein database at the National Center for Biotechnology Information. Multi-sequence alignment was performed using ClustalX2 Multiple Sequence Alignment program. The phylogenetic tree

was constructed by the Neighbor-joining method, using MEGA program (version 6.0; www.megasoftware.net). The cathelicidin sequences used for phylogenetic tree construction contained signal peptide and cathelin domain, the mature peptides were deleted because they were unsuitable for phylogenetic tree construction for their diversity. A total of 1000 bootstrap replicates were used to test the reliability of each branch. The numbers on the branches indicated the percentage of 1000 bootstrap samples supporting the branch.

2.4 Peptide synthesis FM-CATHs were synthesized by thepeptide synthesizer GL Biochem Ltd. (Shanghai, China). The crude peptides were purified by RP-HPLC, and the identity was analyzed by MALDI-TOF MS. Purity of the synthetic peptides was confirmed to be higher than 95%. The physical and chemical parameters of FM-CATHs were shown in Table 2.

2.5 Circular dichroism spectroscopy Circular dichroism (CD) spectroscopy was performed to evaluate the secondary structure of FM-CATHs in solutions. The experiment was carried out using a Jasco J-715 spectrophotometer (Jasco, Japan) according to the method described in our previous paper (Wei *et al.*, 2015). FM-CATHs were dissolved in 0 mM or 60 mM sodium dodecyl sulfate (SDS)/H_2O solutions to an ultimate concentration of 0.2 mg/ml. The spectra at 190–250 nm were measured at 298 K using 0.1 cm path-length cell with 1 nm bandwidth, 1 sec response time, and a scan speed of 100 nm/min. Three consecutive scans per sample were performed and averaged, followed by subtraction of the solvent signal.

2.6 Antimicrobial assay Two-fold broth microdilution method was used to determine the antimicrobial activity of FM-CATHs as described in our previous paper (Ling *et al.*, 2014). 5 Gram-negative bacteria, 5 Gram-positive bacteria and 3 fungi were used in the present study, in which the standard strains were stored in our lab and the clinical strains were collected from the local hospitals. The microbes were incubated in Mueller-Hinton broth (MH broth) at 37°C to exponential phase and diluted with fresh MH broth to 10^6 CFU/ml. Serial dilutions of FM-CATHs (50 μl) were prepared in 96-well microtiter plates and mixed with equal volume of microbe inoculum. The plates were incubated at 37°C for 18 h and the minimal concentrations at which no visible microbial growth occurred were recorded as MIC values. Two traditional antibiotics (ampicillin, meropenem) were used as positive control.

2.7 Bacterial killing kinetic assay In order to evaluate the antimicrobial efficiency of FM-CATHs, bacterial killing kinetic assay was carried out according to the method described previously with minor modifications (Wei *et al.*, 2015). *E. coli* ATCC25922 was incubated in MH broth to exponential phase and diluted to 10^6 CFU/ml with fresh MH broth. FM-CATH1 and FM-CATH2 were added to the bacterial suspension to a final concentration of 5×MIC (46.9 μg/ml). The bacteria were incubated at 37°C for 0, 10, 20, 30, 45, 60, 90, 120, and 180 min. At each time point, 50 μl bacterial solutions were extracted, diluted with fresh MH broth for 1000 times. And 50 μl of the dilutions were coated on MH agar plates, incubated overnight at 37°C, and the viable colonies were counted. Meropenem was used as positive control and sterile deionized water was used as blank control.

2.8 Cytotoxic and hemolytic assay MTT [3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] method was used to determine the cytotoxic activities of FM-CATHs on two mammalian tumor cell lines (human liver hepatocellular carcinoma cell line HepG2, human prostate cancer cell line PC3) and one normal mammalian cell line (mice fibroblast cell line L929) (Ling *et al.*, 2014). Briefly, the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C. Cells (approximately 2×10^4 per well in 100 μl medium) were seeded in 96-well plates and cultured overnight until adhered to the plate. Various concentrations of FM-CATHs dissolved in DMEM medium were added to the wells and the plates were incubated at 37°C for 48 h. The subsequent procedure was performed according to the standard MTT method. Cell death induced by FM-CATHs was expressed as the percentage to the blank control group, which was regarded as 100%.

To determine the cytotoxic activities of FM-CATHs on human erythrocytes, hemolytic assay was performed as previously reported (Ling *et al.*, 2014). 1% Triton X-100 (v/v) was used to determine the 100% hemolysis and 0.9% saline was used as blank control.

2.9 Cytoplasmic membrane permeabilization assay The method for determination of bacterial membrane permeability was adapted from the method with minor modifications (Rawlinson *et al.*, 2010). *E. coli* ATCC25922 and *B. subtilis* (CI) were cultured in MH broth to exponential phase and washed with sterile PBS for three times. The bacteria were stained with calcein-AM (5 μM in PBS, Yeasen, Shanghai, China) for 90 min

at 37°C and excessive stains were removed by washing with PBS for three times. The cells were resuspended with PBS (1×10^8 CFU/ml) and FM-CATHs were added to a final concentration of 5×MIC. The culture was incubated at 37°C for 2 h, and then centrifuged at 6000 rpm for 10 min. The calcein leakage to the supernatant was recorded with fluorescent microplate autoreader (excitation at 490 nm and emission at 515 nm). 100% calcein leakage was obtained by incubating bacteria with 1% Triton X-100 (v/v).

2.10 Inhibition of biofilm formation assay The ability of FM-CATHs in inhibiting the formation of bacterial biofilms was determined according to the method described in other papers with minor modifications (De Zoysa *et al.*, 2015). The bacterium used in the experiment was a clinically isolated *E. coli* strain, which had been proved possessing the ability to form biofilms. The MIC value of FM-CATH1 and FM-CATH2 against the bacterium was 18.75 μg/ml. Briefly, the bacteria were incubated in LB broth to exponential phase and diluted to 2×10^7 CFU/ml. Serial dilutions of FM-CATHs (16, 8, 4, 2, 1, 0.5 μg/ml) were prepared with LB broth in polystyrene 96 well microtiter plates (50 μl). Equal volumes of bacterial dilutions were added to the wells, and the plates were statically incubated at 37°C for 48 h. At the end of incubation, supernatants were discarded and the wells were washed with sterile PBS (pH 7.4) for three times. 100 μl of 1% crystal violet (dissolved in PBS) was added to each well to stain for 30 min. After that, the stain was removed, and the wells were washed with sterile water for three times. The stained biofilms at the bottom of each well were dissolved with ethanol and absorbance at 560 nm was recorded for a semiquantitative estimation of biofilm biomass.

2.11 Anti-inflammatory assay Mouse peritoneal macrophages (MPMs) from C57 mice were prepared according to the previous paper (Wei *et al.*, 2015). After collection, MPMs were cultured in RPMI-1640 medium (containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, Gibco) and plated in 96-well culture plate (1×10^4 cells/well). After cell adhersion, the culture medium was replaced with fresh RPMI-1640 medium containing 2% FBS. LPS (from *E. coli* 055:B5, Sigma-Aldrich, USA) and FM-CATHs were added to MPMs to ultimate concentrations of 100 ng/ml and 10 μg/ml, respectively. After a 6 h-incubation, the culture supernatants and cells were collected.

Total RNA of the cells were extracted by Trizol reagent (Life Tech, USA) and cDNAs were synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan). The quantitative real-time PCR (qRT-PCR) experiment was performed using a SYBR Premix Ex Taq™ II (Tli RNaseH Plus) two-step qRT-PCR kit (Takara, Japan) on an ABI Prism 7000 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The cycle counts of TNF-α, IL-1β and IL-6 genes were normalized to GAPDH gene, and accordingly the fold changes of the target genes were calculated. The primers used for qRT-PCR were listed in Table 1.

The production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in culture supernatants was determined with ELISA (eBiosciences, USA) according to the kit instructions.

2.12 Ethical statement The present study involving human participants and animals was approved by the Institutional Ethics Committee of Weifang University of Science and Technology before the study began. All procedures performed in this study were according to the Standard Operation Procedures of the Guide for the Use of Experimental Animals of Weifang University of Science and Technology. Frog collection and tissue preparation procedures were approved by the Institutional Animal Care and Use Committee of Weifang University of Science and Technology (Permit Number: 20140710). Anti-inflammatory experiment using C57 mice was approved by the Institutional Animal Care and Use Committee of Weifang University of Science and Technology (Permit Number: 20160305). Written informed consent was obtained from the blood donor to have his blood drawn and used in this study.

3. Results

3.1 Identification and evolutionary analysis of FM-CATHs By PCR-based gene cloning, two cDNAs encoding two novel cathelicidins termed FM-CATH1 and FM-CATH2 were obtained from the constructed lung cDNA library of *F. multistriata* (GenBank™ accession numbers: KX495359–KX495360). As shown in Figure 1, the cDNAs encoding FM-CATH1 and FM-CATH2 precursors were 447 bp in length, and the translated FM-CATHs precursors comprised 148 amino acid residues. Multi-sequence alignment of cathelicidins revealed that consistent with other representative cathelicidins, FM-CATHs precursors comprised an N-terminal signal peptide sequence, a highly conserved cathelin domain and a variable C-terminal mature peptide sequence (Figure 2). Although FM-CATHs precursors shared relatively low similarity with cathelicidins from mammals and reptiles,

FM-CATH1

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atgaaggtctggcagtgtgtgctctggatctgcgcgatcacattgcactcagctcgctcg   60
 M  K  V  W  Q  C  V  L  W  I  C  A  I  T  L  H  S  A  R  S    20
cagtcctcagatcaggacgggtggatcagagaggccttggatctctacaaccagaaggat   120
 Q  S  S  D  Q  D  G  W  I  R  E  A  L  D  L  Y  N  Q  K  D    40
gatggggagttctgctttaaattcctgtcggatctcccagatgcccttctggaggaggag   180
 D  G  E  F  C  F  K  F  L  S  D  L  P  D  A  L  L  E  E  E    60
ggagactctcaatctatcggcttcctaataaaggagacggactgcctgaaatctgaaggc   240
 G  D  S  Q  S  I  G  F  L  I  K  E  T  D  C  L  K  S  E  G    80
caagacttggagcaatgcgactacaaggaggacggggaggtgaaggcctgcgttctgagc   300
 Q  D  L  E  Q  C  D  Y  K  E  D  G  E  V  K  A  C  V  L  S   100
gcagaagaggaggtgaagtgcgtcagcctgtctgagaagcgacgcacccggagagccatc   360
 A  E  E  E  V  K  C  V  S  L  S  E  K  R  R  T  R  R  A  I   120
aaaaaactgaaaaccaaagtcttaaacaaactgaagcaaaagctccaagctgtcggcaat   420
 K  K  L  K  T  K  V  L  N  K  L  K  Q  K  L  Q  A  V  G  N   140
ctcatcgggagcgtgatcaaagcataaatgaacatccgctgcagaaaaaacgcttcaatc   480
 L  I  G  S  V  I  K  A  *                                     148
cgatcatcgcaatcagttatatccagaggaaaggaaaccgcaatatacgtcccccgcact   540
tcttattagcaaatattgtgtgtgcactgaatacacagatttatcaataaattcagtacc   600
atttgctttaaaaaaaaaaaaaaaaa                                     626
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FM-CATH2

```
atgaaggtctggcagtgtgtgctctggatctgcgcgatcacattgcactcagctcgctcg   60
 M  K  V  W  Q  C  V  L  W  I  C  A  I  T  L  H  S  A  R  S    20
cagtcctcagatcaggacgggtggatcagagaggccttggatctctacaaccagaaggat   120
 Q  S  S  D  Q  D  G  W  I  R  E  A  L  D  L  Y  N  Q  K  D    40
gatggggagttctgctttaaattcctgtcggatctcccagatgcccttctggaggaggag   180
 D  G  E  F  C  F  K  F  L  S  D  L  P  D  A  L  L  E  E  E    60
ggagactctcaatctatcggcttcctaataaaggagacggactgcctgaaatctgaaggc   240
 G  D  S  Q  S  I  G  F  L  I  K  E  T  D  C  L  K  S  E  G    80
Caagacttggagcaatgcgactacaaggaggacggggaggtgaaggcctgcgttctgagc   300
 Q  D  L  E  Q  C  D  Y  K  E  D  G  E  V  K  A  C  V  L  S   100
gcagaagaggaggtgaagtgcgtcagcctgtctgagaagcgacgcacccggagagccatc   360
 A  E  E  E  V  K  C  V  S  L  S  E  K  R  R  T  R  R  A  I   120
aaaaaactgaaaaccaaagccttaaacaaactgaagcaaaagctccaagctgtcggcaat   420
 K  K  L  K  T  K  A  L  N  K  L  K  Q  K  L  Q  A  V  G  N   140
ctcatcgggagcgtgatcaaaggataaatgaacatccgctgcagaaaaaacgcttcaatc   480
 L  I  G  S  V  I  K  G  *                                     148
cgatcatcgcaatcagttatatccagaggaaaggaaaccgcaatatacgtcccccgcact   540
tcttattagcaaatattgtgtgtgcactgaatacacagatttatcaataaattcagtacc   600
atttgctttataaaaaaaaaaaaaaaaaaa                                 630
```

Figure 1 The cDNA sequences encoding FM-CATHs and the predicted precursor sequences. The putative mature peptides of FM-CATHs are boxed. The stop codon is indicated by an asterisk (*).

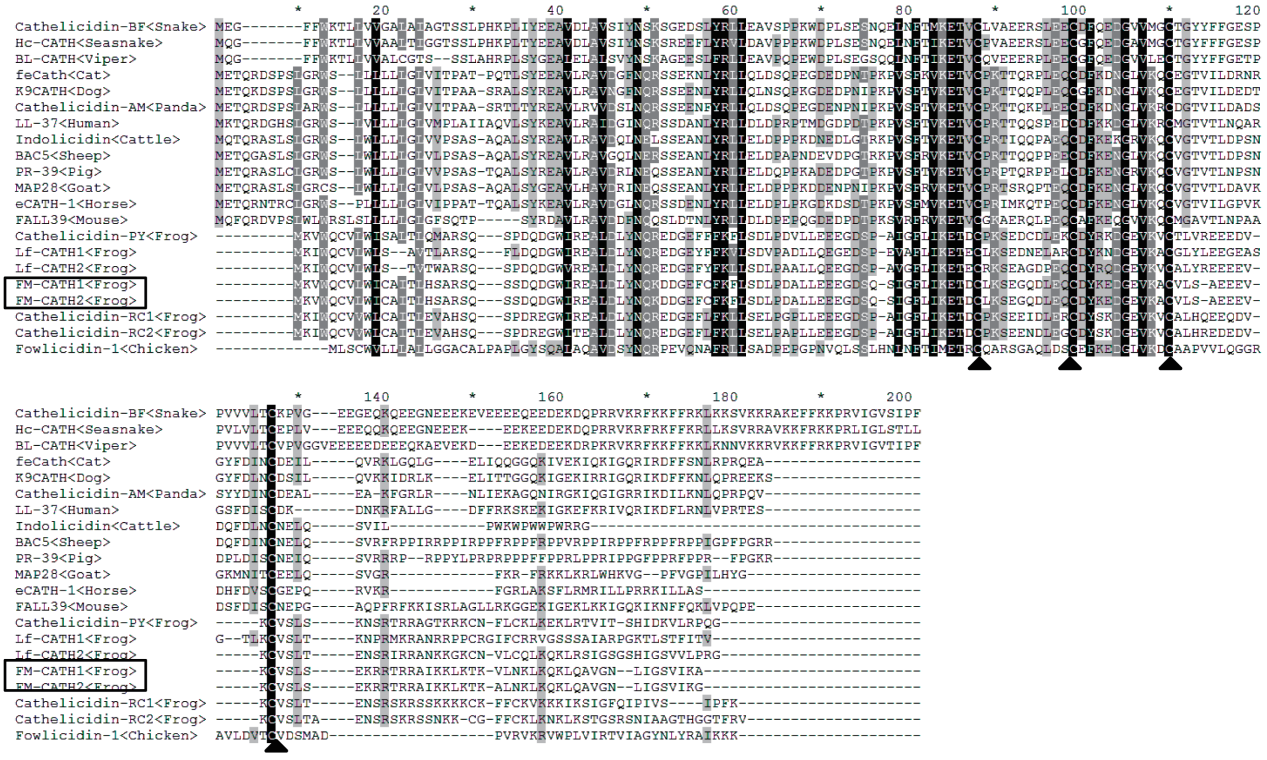

Figure 2 Multi-sequence alignment of FM-CATHs with other representative cathelicidins.

Table 1 Primers (mouse) used for qRT-PCR.

Name	Forward (5'-3')	Reverse (3'-5')
TNF-α	CGGTGCCTATGTCTCAGCCT	GAGGGTCTGGGCCATAGAAC
IL-1β	ATGGCAACTGTTCCTGAACTC	GCCCATACTTTAGGAAGACA
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
GAPDH	GTGAAGGTCGGTGTGAACGGATT	GGAGATGATGACCCTTTTGGCTC

Table 2 Physical and chemical parameters of FM-CATH1 and FM-CATH2.

Peptide	GRAVY	Number of amino acids	Net charge	*Theoretical pI*	*Mw*
FM-CATH1	–0.329	34	11+	12.33	3815.7
FM-CATH2	–0.465	34	11+	12.33	3773.6

they exhibited high degree of similarity with other frog cathelicidins. Especially, the highly conserved four cysteines at the end of cathelin domain in all cathelicidins invariantly existed in the FM-CATHs precursors. According to the amino acid sequences of known frog cathelicidins, the mature peptides of FM-CATHs were predicted (Figure 1). FM-CATH1 and FM-CATH2 are composed of 34 amino acid residues, their sequences were RTRRAIKKLKTKVLNKLKQKLQAVGNLIGSVIKA and RTRRAIKKLKTKALNKLKQKLQAVGNLI GSVIKG, respectively. Their physical and chemical parameters were shown in Table 2. Sequence BLAST with NCBI protein database indicated that FM-CATH1 and FM-CATH2 showed no similarity with the known cathelicidins, including the cathelicidins from other frogs. Using the neighbor-joining method, we constructed a phylogenetic tree of cathelicidins (Figure 3). Accordingly, the cathelicidins were divided into four major clusters, *i.e.* cathelicidins from mammals, reptiles, birds, and frogs. As we expected, FM-CATHs clustered with the other five frog cathelicidins, which implied that FM-CATHs and the other frog cathelicidins may originate from a common ancestor gene.

3.2 Secondary structure of FM-CATHs As shown in Figure 4A and 4B, the main region of FM-CATH1 (Thr-2–Lys-33) and FM-CATH2 (Thr-2–Ile-32) were predicted to adopt an alpha-helical conformation. The software package provided by the ExPASy proteomics was used

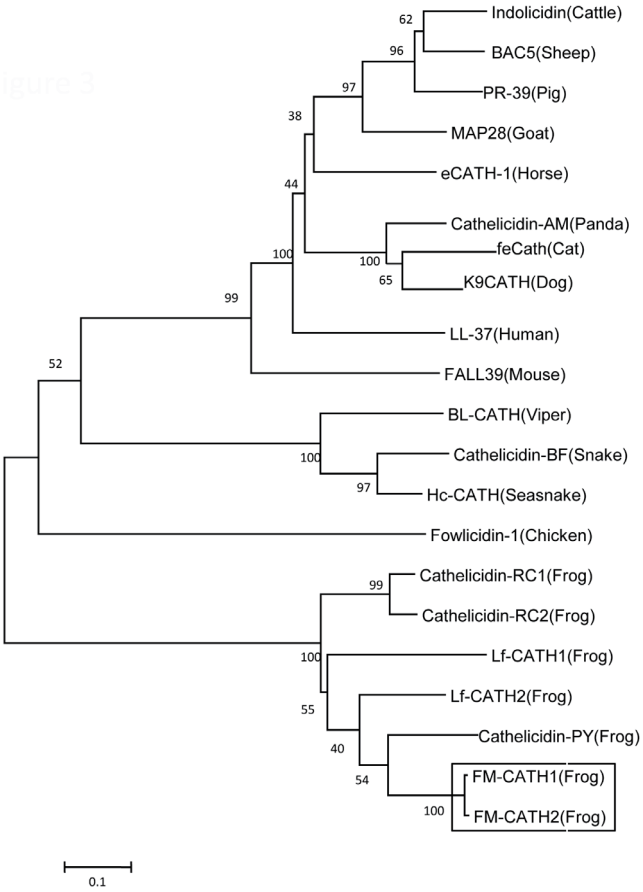

Figure 3 Phylogenetic analysis of cathelicidins. The tree was constructed by the neighbor-joining method based on the proportion difference of the signal peptide and cathelin domain. A total of 1000 bootstrap replicates were used to test the reliability of each branch. The numbers on the branches indicate the percentage of 1000 bootstrap samples supporting the branch. Only branches supported by a bootstrap value of at least 50% are shown. FM-CATHs are boxed.

to estimate the amphipathicity of FM-CATH1 and FM-CATH2, the results were shown in Figure 4C and 4D, respectively. A helix-wheel diagram of the N-terminal alpha-helix region of FM-CATH1and FM-CATH2 (Thr-2–Lys-33 and Thr-2–Ile-32) was plotted. The hydrophobic amino acids were shown in yellow and gray, concentrating on one side of the helix. The hydrophilic amino acids were shown in blue and purple concentrating on the other side of the helix. This amphipathic alpha-helical conformation is universal for most of AMPs, which is generally believed to be directly relevant to their antimicrobial function (Nakatsuji and Gallo, 2012; Brogden, 2005).

The secondary structures of FM-CATH1 and FM-CATH2 in solvents were further confirmed by CD spectroscopy (Figure 4E and 4F).The CD spectra of both FM-CATH1 and FM-CATH2 dissolved in H_2O showed a strong negative peak at 200nm, indicating that they adopted a random-coil conformation in H_2O. However, in the membrane-mimetic environment of 60 mM SDS/H_2O solution, the CD spectra of FM-CATH1 and FM-CATH2 showed a strong positive peak at 190 nm and two negative peaks at 208 nm and 222 nm, indicating that FM-CATH1 and FM-CATH2 mainly adopt alpha-helical conformation in membrane-mimetic environments.

3.3 Antimicrobial activity of FM-CATHs FM-CATH1 and FM-CATH2 were chemically synthesized and the purity was confirmed to be >95%. The MICs of the two peptides against 13 microorganisms, including Gram-positive bacteria, Gram-negative bacteria and fungi were determined. The results were listed in Table 3. Both FM-CATH1 and FM-CATH2 exhibited potent and broad-spectrum antimicrobial activity against all of the tested 13 microorganisms, with MICs ranging from 1.23 to 19.87 μM. Moreover, 6 strains of the 13 microorganisms were ampicillin-resistant, but FM-CATH1 and FM-CATH2 could effectively kill them at low concentrations. As expected, the positive control meropenem, a new generation of carbapenem antibiotic, was more effective

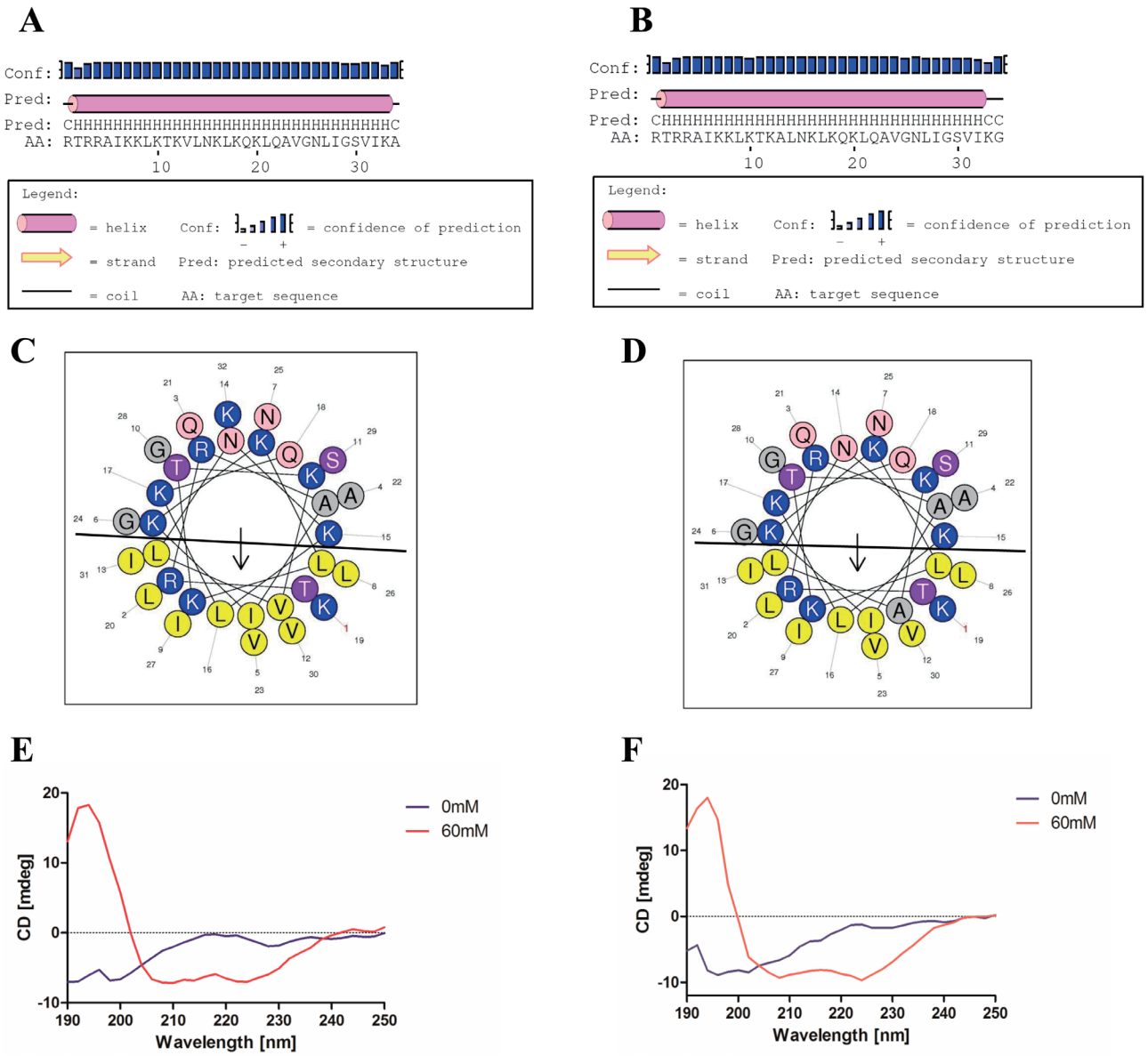

Figure 4 Secondary structure prediction and analysis of FM-CATHs. A and B, secondary structures of FM-CATH1 and FM-CATH2 predicted by the PSIPRED protein structure prediction server provided by the Bioinformatics Group of the Department of Computer Science, University College London. C and D, helix-wheel diagrams of FM-CATHs alpha-helical region plotted by the software package of the ExPASy proteomics server. Hydrophobic amino acids are shown in yellow and gray. Hydrophilic amino acids are shown in blue and purple. E and F, Circular dichroism analysis of FM-CATHs in different solvent environments. Blue, FM-CATH1 dissolved in H_2O. Red, FM-CATH1 dissolved in 60 mM SDS/H_2O solution.

than FM-CATHs, with MICs ranging from 0.03 to 24.45 μM. But for *Enterococcus faecium* clinical strain, FM-CATH1 and FM-CATH2 were more effective than meropenem, with MICs of 4.91 μM and 4.97 μM, respectively, while the MIC of meropenem was 24.45 μM.

3.4 Bacterial killing kinetics of FM-CATHs To examine the antimicrobial efficiency of FM-CATHs, bacterial killing kinetic assay was performed using colony counting method. The results were illustrated in Table 4. At the concentration of 5×MIC, FM-CATH1 and FM-CATH2 exhibited a rapid killing effect on the tested *E. coli* ATCC25922 within 60 min. It is worth noting that even if the incubation time extended to 180 min, the colony forming units (CFUs) remained zero, which implied that the antimicrobial property of FM-CATH1 and FM-CATH2 was bactericidal rather than bacteriostasic. In contrast, although the antimicrobial activity of meropenem was more potent than FM-CATHs, it needed at least 120 min to completely eliminate *E. coli*

Table 3 Antimicrobial activity of FM-CATH1 and FM-CATH2.

Microorganisms	MIC (μg/ml)			
	FM-CATH1	FM-CATH2	Meropenem	Ampicillin
Gram-negative bacteria				
Escherichia coli ATCC25922	9.38 (2.46 μM)	9.38 (2.49 μM)	0.01 (0.03 μM)	4.69 (12.62 μM)
Shigella dysenteriae clinical strain	4.69 (1.23 μM)	9.38 (2.49 μM)	0.03 (0.08 μM)	75 (201.9 μM)
Klebsiella peneumoniae clinical strain	37.5 (9.83 μM)	75 (19.87 μM)	0.06 (0.15 μM)	>200
Acinetobacter baumannii clinical strain	37.5 (9.83 μM)	37.5 (9.94 μM)	0.23 (0.61 μM)	37.5 (100.9 μM)
Pseudomonas aeruginosa ATCC27853	9.38 (2.46 μM)	18.75 (4.97 μM)	0.06 (0.15 μM)	>200
Gram-positive bacteria				
Staphylococcus aureus ATCC25923	37.5 (9.83 μM)	18.75 (4.97 μM)	0.06 (0.15 μM)	9.38 (25.24 μM)
Bacillus cereus clinical strain	18.75 (4.91 μM)	37.5 (9.94 μM)	0.06 (0.15 μM)	>200
Bacillus subtilis clinical strain	9.38 (2.46 μM)	9.38 (2.49 μM)	0.03 (0.08 μM)	75 (201.9 μM)
Enterococcus faecium clinical strain	18.75 (4.91 μM)	18.75 (4.97 μM)	9.38 (24.45 μM)	>200
Nocardia asteroides clinical strain	37.5 (9.83 μM)	75 (19.87 μM)	4.69 (12.22 μM)	18.75 (50.49 μM)
Fungi				
Candida albicans clinical strain 1	18.75 (4.91 μM)	37.5 (9.94 μM)	0.12 (0.31 μM)	>200
Candida albicans clinical strain 2	37.5 (9.83 μM)	37.5 (9.94 μM)	0.23 (0.61 μM)	>200
Candida glabrata clinical strain	18.75 (4.91 μM)	18.75 (4.97 μM)	0.03 (0.08 μM)	18.75 (50.49 μM)

MIC: minimal inhibitory concentration. These concentrations represent mean values of three independent experiments performed in duplicates.

Table 4 Killing kinetics of FM-CATH1 and FM-CATH2 against *E. coli* ATCC25922.

Time	Colony Forming Units (×103, CFUs/ml)								
	0 min	10 min	20 min	30 min	45 min	60 min	90 min	120 min	180 min
FM-CATH1	76	60.3	51	16	2	0	0	0	0
FM-CATH2	82.7	61.3	50.7	20.7	5.7	0	0	0	0
Meropenem	70.7	87	83.3	74.7	61	54.7	3.3	0	0
Control	81.7	81	92	101	118.7	172	375.7	692.7	1180

E. coli ATCC25922 was mixed with samples at concentration of 5×MIC for 0, 10, 20, 30, 45, 60, 90, 120 and 160 mins. The results represent mean values of three independent experiments performed in duplicates.

ATCC25922 cells, which was much slower than FM-CATHs

3.5 Cytotoxicity and hemolysis of FM-CATHs Two mammalian tumor cell lines (human liver hepatocellular carcinoma cell line HepG2, human prostate cancer cell line PC3) and one mammalian normal cell line (mice fibroblast cell line L929) were used to evaluate the cytotoxicity of FM-CATH1 and FM-CATH2. As illustrated in Table 5, the two peptides exhibited relatively low cytotoxic activity against the tested cell lines. At a concentration up to 200 μg/ml, FM-CATH1 induced 14.5%, 13.6% and 21.3% death of HepG2, PC3 and L929 cells, respectively. While at the same concentration, FM-CATH2 induced 16.5%, 14.6% and18.9% death of HepG2, PC3 and L929 cells, respectively.

In this study, human fresh erythrocytes were also used to evaluate the hemolytic activity of FM-CATH1 and FM-CATH2. As illustrated in Table 5, at a concentration of 200 μg/ml, FM-CATH1 and FM-CATH2 induced 15.2% and 16.8% human erythrocyte hemolyisis, respectively.

3.6 Effect of FM-CATHs on microbial membrane integrity *E.coli* ATCC25922 and *B. subtilis* (CI) were used to evaluate the effect of FM-CATHs on microbial membrane integrity. As shown in Figure 5, after incubated for 2 h at a concentration of 5×MIC, FM-CATH1 and FM-CATH2 induced 62.8% and 61.3% calcein release, repectively for *E.coli* ATCC25922. Under the same condition, FM-CATH1 and FM-CATH2 induced 44.6% and 42.7% calcein release, repectively for *B. subtilis*(CI) (Figure 5). The results indicated that FM-CATH1 and FM-CATH2 can significantly induce the disruption of microbial membrane integrity and a higher membrane permeability, which ultimately resulted in the leakage of cellular inclusions and cell death.

3.7 Inhibition of bacterial biofilm formation Biofilms enable bacteria to survive and spread in the environment outside the host and even are resistant to high concentrations of antibiotics (De Zoysa *et al.*, 2015; Janssens *et al.*, 2008). Therefore, the ability of FM-CATHs in inhibiting the formation of biofilms of *E. coli*

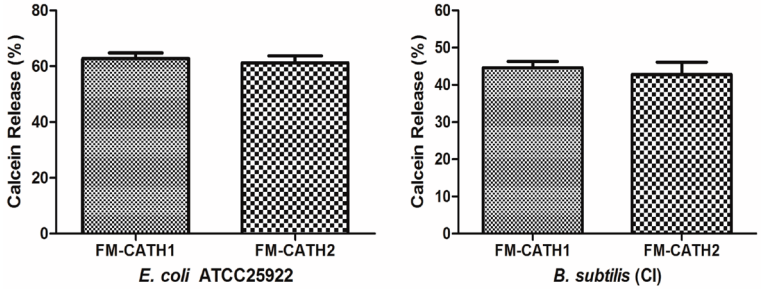

Figure 5 Calcein release induced by FM-CATH1 and FM-CATH2. (A) *E. coli* ATCC25922; (B) *B.subtilis* (CI).

(CI) was investigated. As shown in Figure 6, FM-CATHs exhibited a dose-dependent effect on the formation of *E. coli* (CI) biofilms at concentrations below 1×MIC (18.75 μg/ml). For example, at a concentration of 16 μg/ml, FM-CATH1 and FM- CATH2 inhibited 62.2% and 65.4% bacterial biofilm formation, respectively.

3.8 FM-CATHs inhibit LPS-induced pro-inflammatory cytokine transcription and production

As an important kind of pathogen-associated molecular pattern molecules (PAMPs), LPS induces the activation of TLR4 inflammatory signaling pathway and promotes the production of pro-inflammatory cytokines. In the present study, we studied the anti-inflammatory activities of FM-CATHs by evaluating the effect of FM-CATHs on LPS-induced pro-inflammatory cytokine transcription and production in MPM cells. As shown in Figure 7, FM-CATHs alone (10 μg/ml) did not alter the gene transcription and protein production of TNF-α, IL-1β and IL-6. However, FM-CATHs (10 μg/ml) significantly inhibited the transcription and production of TNF-α, IL-1β and IL-6 induced by LPS (100 ng/ml). 10 μg/ml FM-CATHs inhibited the expression of TNF-α, IL-1β and IL-6 genes by >50%. Similarly, 10 μg/ml FM-CATHs inhibited the LPS-induced TNF-α, IL-1β and IL-6 production by >45%. All these results indicated the potent anti-inflammatory activities of FM-CATHs.

4. Discussion

In recent years, the abuse of antibiotics has resulted in more and more severe microbial drug-resistance, which seriously threatens the public health. As a result, the requirement for novel antimicrobial agents becomes urgent. In 1980s, the first antimicrobal peptide (AMP) was separated and studied (Steiner *et al.*, 1981). From then on, hundreds of AMPs were identified (Nakatsuji and Gallo, 2012; Brogden, 2005). The characteristics of small size, good stablity, broad-spectrum and potent antimicrobial activity, special mechanism and slight possibility to induce resistance make AMPs good candidates for the development of novel peptide antibiotics (Hancock and Sahl, 2006). So far, dozens of AMPs and their analogs have been applied to commercial development (Steinstraesser *et al.*, 2011). Among them, three cathelicidin analogs are undergoing clinical trials. The cattle indolicidin analogue omiganan (MBI-226, CPI226) is undergoing Phase 3 clinical study in healthy adult subjects for treatment of topical skin sepsis and local catheter site infection in patients with central venous catheters (Isaacson, 2003). Another analogue of indolicidin named MX-594AN, is undergoing Phase 2 clinical trial for the treatment of papulopustular rosacea (Steinstraesser *et al.*, 2011). In addition, pig protegrin-1 analogue iseganan (IB-367) is undergoing Phase 3 clinical trial on the prevention of oral mucositis for patients undergoing chemotherapy due to head and neck cancer (Elad *et al.*, 2012). Therefore, identifying novel cathelicidins from vertebrates is necessary and meaningful for peptide antibiotic design.

In the present study, two novel cathelicidins (FM-CATH1 and FM-CATH2) were identified from the lung of frog *F. multistriata*. The cloned cDNAs encoding FM-CATH1 and FM-CATH2 were 447 bp in length (Figure 1). And the translated precursors contained 148 amino acid residues. Consistent with other cathelicidins, the precursors of FM-CATHs included an N-terminal signal peptide, a highly conserved cathelin domain and a C-terminal variable mature peptide (Figure 2). And the signal peptide sequence and cathelin domain of FM-CATHs precursors showed high sequence similarity with other cathelicidins from frogs, but the mature peptides varied greatly. Using the signal peptide and cathelin domain sequences of representative cathelicidins deposited in NCBI database, a phylogenetic tree of cathelicidins was constructed (Figure 3). Accordingly, FM-CATHs and the other frog cathelicidins, including cathelicidin-RC, cathelicidin-PY and Lf-CATH, were grouped together in a sub-ordinate branch, which indicates

Table 5 Cytotoxicity and Hemolysis of FM-CATH1 and FM-CATH2.

Cells	Mortality/hemolytic rate (%)	
	FM-CATH1	FM-CATH2
HepG2	14.5	16.5
PC3	13.6	14.6
L929	21.3	18.9
Human Erythrocytes	15.2	16.8

The ultimate concentration of FM-CATHs in the assay was 200 μg/ml. The results represent mean values of three independent experiments.

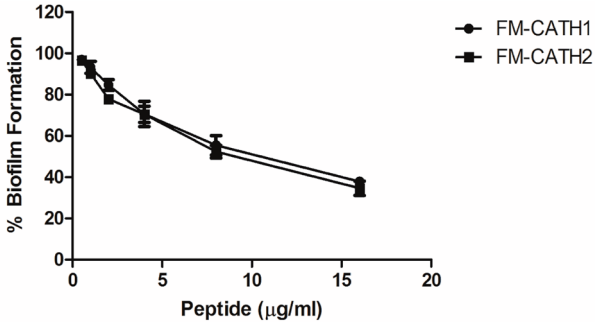

Figure 6 Inhibitory effect of FM-CATHs on the formation of bacterial biofilms of *E.coli* (CI). The concentrations of FM-CATHs used in the experiment were 16, 8, 4, 2, 1, 0.5 μg/ml, respectively.

that they may evolve from the same ancestor gene. From the results of previous studies, two cathelicidins with significant sequence difference usually can be explored from one frog species (Ling *et al.*, 2014; Wei *et al.*, 2012; Yu *et al.*, 2013). However, the identified two cathelicidins in the present study possess great sequence similarity, with merely two amino acid differences.

FM-CATHs were chemically synthesized and their advanced structures were studied using bioinformatics prediction and CD investigation. As predicted, FM-CATH1 and FM-CATH2 mainly assumed an amphipathic alpha-helical conformation, which was the most common spatial arrangement among cathelicidin family AMPs (Figure 4) (Wong *et al.*, 2013). And the results of CD experiment further indicate that in H_2O, FM-CATH1 and FM-CATH2 adopt a random-coil conformation, but in the membrane-mimetic environment of 60 mM SDS/H_2O solutions, FM-CATH1 and FM-CATH2 change their conformation into alpha-helix to facilitate their insertion into microbial membrane (Figure 4).

The results of antimicrobial assay indicate that FM-CATH1 and FM-CATH2 possess potent and broad-spectrum antimicrobial activity against the tested microorganisms (Table 3). All the tested 13 microorganism strains were sensitive to FM-CATH1 and FM-CATH2 with MICs ranging from 1.23 to 19.87 μM. And theantimicrobial activity of FM-CATH1 was comparative to FM-CATH2, due to their great sequence similarity. It is worth noting that 6 of the 13 tested microorganisms are ampicillin-resistant, which implies the great potential of FM-CATHs in the treatment of infections induced by drug-resistant pathogens.

Furthermore, the bacterial killing kinetic results indicate that the bacterial killing effect of FM-CATH1 and FM-CATH2 are rapid. They could kill the tested *E. coli* ATCC25922 cells in 60 min, which was far more rapid than the positive control meropenem (at least 120 min) (Table 4). Although the antimicrobial activities of FM-CATHs were less potent than meropenem (Table 3), the more rapid bacterial killing action provides a remedy for their development as novel antimicrobial agents. It has been reported that many cathelicidins kill microbial cells through permeabilization of the cytoplasmic membrane (Wang *et al.*, 2011; Wei *et al.*, 2012). The increasement of membrane permeabilization causes the disruption of microbial cell integrity, which ultimately results in the leakage of cellular inclusions and cell death. According to the results of calcein release experiment, FM-CATH1 and FM-CATH2 could significantly induce the calcein release in both Gram-positive and Gram-negative bacteria (Figure 5). The rapid killing effect and induction of calcein release indicates that FM-CATH1 and FM-CATH2 target bacterial membrane and induce permeabilization of the membrane, which ultimately results in bacteria cell death.

It has been realized for many years that most bacteria grow predominantly as biofilm rather than as free-living planktonic cells in diverse environments (Hall-Stoodley *et al.*, 2004). Biofilm provides effective protect to the bacteria from external stress factors, such as antimicrobial agents and host immune responses (Davies, 2003). As a result, drugs with potent biofilm inhibitory activities have great potential in dealing with infectious diseases induced by drug-resistant bacteria. In the present study, we examined the effect of FM-CATHs on the formation of bacterial biofilms. To our surprise, we found that FM-CATH1 and FM-CATH2 can significantly inhibit the formation of bacterial biofilms at low concentrations below 1×MIC. This suggests s great value for FM-CATHs to be developed as novel anti-infective agents against drug-resistant bacteria.

Besides direct antimicrobial and biofilm inhibitory activities, FM-CATHs also exhibited potent anti-inflammatory activities in an LPS-induced inflammatory cell model. At a concentration of as low as 10 μg/ml, both FM-CATH1 and FM-CATH2 significantly inhibited the LPS-induced gene transcription and protein production

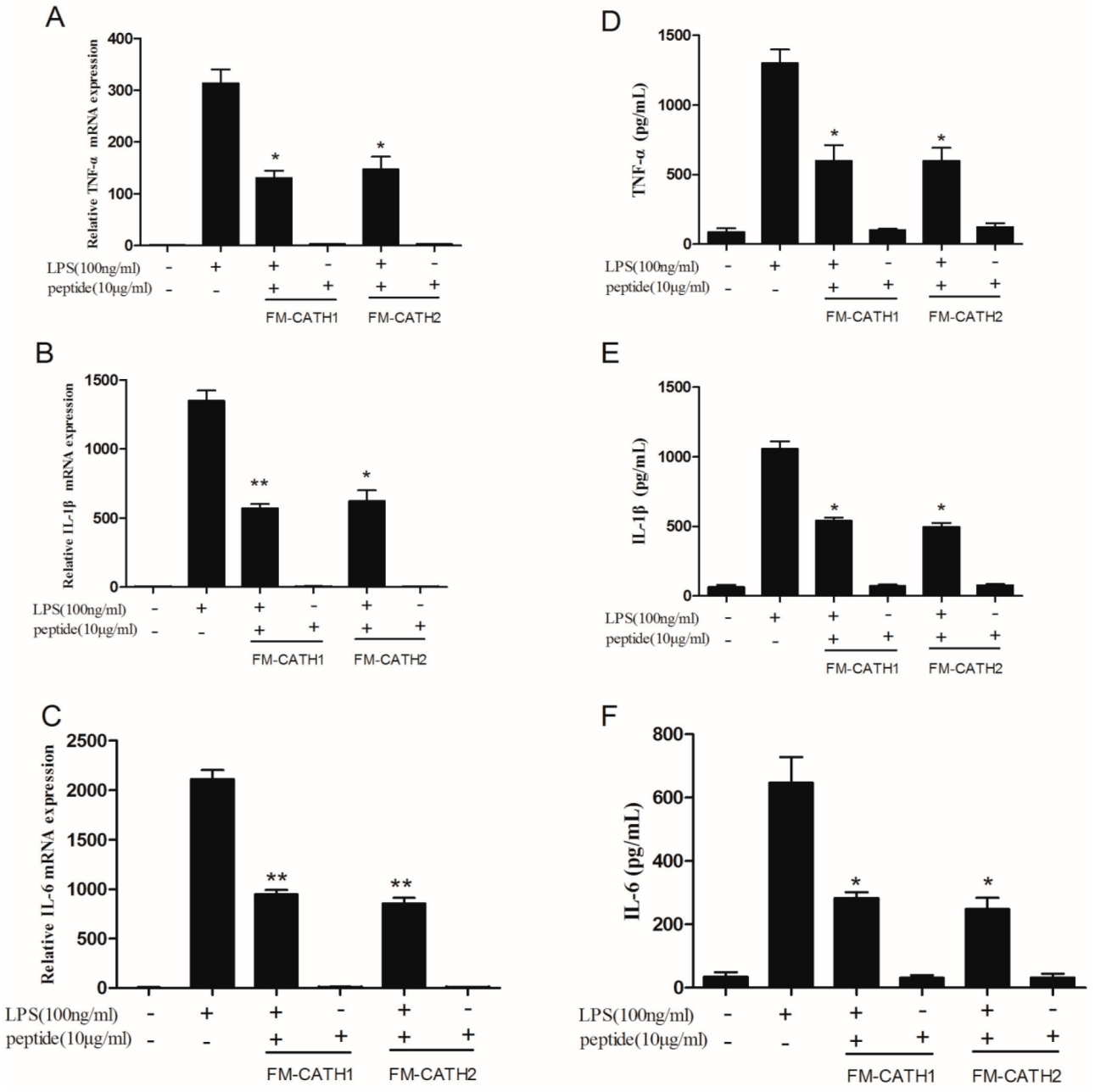

Figure 7 Effect of FM-CATHs on LPS-induced pro-inflammatory cytokines transcription and production in MPM cells. A, TNF-α mRNA. B, IL-1β mRNA. C, IL-6 mRNA. D, TNF-α production. E, IL-1β production. F, IL-6 production. Data are mean ± SEM value of three separate experiments. $*P < 0.05$, $**P < 0.01$ significantly different compared to the control.

of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in MPMs (Figure 7). The results above imply that FM-CATHs possess multiple functions in frog host. At high concentrations, they protect the host with their direct antimicrobial activities and biofilm inhibitory activities. While at low concentrations, they modulate the host immunity to protect against bacterial infections.

Besides, we also examined the cytotoxicity and hemolysis of FM-CATHs. At a concentration up to 200 μg/ml, FM-CATHs exhibited relatively low cytotoxic activities toward the tested mammalian cells, implying their promising therapeutic potential against microbial infections.

In conclusion, our study reported the identification and characterization of two novel cathelicidin family AMPs, FM-CATH1 and FM-CATH2, from the frog *F. multistriata*. Both FM-CATH1 and FM-CATH2 exhibited low sequence similarity with the known cathelicidins.

FM-CATH1 and FM-CATH2 exhibited potent, broad-spectrum, and rapid antimicrobial activity. They killed bacteria through inducing the permeabilization of bacterial membrane. They also exhibited significant inhibitory effect on the formation of bacterial biofilms at low concentrations below 1×MIC. Besides, they exhibited potent anti-inflammatory activities at low concentrations. Furthermore, they showed relatively low cytotoxic effect toward mammalian cells. All of these properties make FM-CATH1 and FM-CATH2 potent templates for the development of novel peptide antibiotics.

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